# THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JAN 2 5 1974

Application for Research Grant
(Use extra pages as needed)

Date: January 10, 1974

1. Principal Investigator (give title and degrees):

Allen Barry Cohen, M.D., Ph.D.
Assistant Professor of Medicine in Residence

2. Institution & address:

University of California Service
Chest Division
San Francisco General Hospital
San Francisco, California 94110

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

4. Short title of study:

The Genetic Defect in Alpha-1-Antitrysin Deficient Patients

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

The occurrence of alpha-1-antitrypsin deficiency in some patients with familial emphysema is one of the few clues available to the nature of the biochemical processes in human emphysema. The purpose of these experiments is to determine the biochemical basis for the genetically determined differences between the alpha-1-antitrypsin of normal subjects and that of subjects with the genetic variant of alpha-1-antitrypsin which predisposes some of them to emphysema. These studies may enable us to determine whether the abnormality in alpha-1-antitrypsin is the primary defect in these individuals or whether there are other defects as well. In addition, if it can be concluded that other defects are likely to exist, this investigation may suggest experiments to discover those defects.

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8. Brief statement of working hypothesis:

The purpose of these experiments is to differentiate between two alternative hypotheses:

A) The abnormal gene which leads to a deficiency of alpha-1-antitrypsin in the blood is the structural gene for alpha-1-antitrypsin which codes for the alpha-1-antitrypsin polypeptide.

B) The abnormal gene controls some aspect of alpha-1-antitrypsin other than the primary sequence of amino acids such as the number and type of charged carbohydrate moieties on the molecule.

9. Details of experimental design and procedures (append extra pages as necessary)

Please see attached pages, beginning at Page 7.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities:

Laboratories at the San Francisco General Hospital, Building 100, Room 272 and 273. (Room 272 is on loan for an indefinite time.)

Space:

Room 272 = 185 sq.ft. Room 273 = 480 sq. ft.

Equipment:

Centrifuges: Sorval RC3, Beckman Model L3-50 preparative ultracentrifuge

Electrophoretic equipment: Immunoelectrophoresis, starch block, pevikon block, preparative polyacrylamide gel, analytic polyacrylamide gel, thin layer, and two current or voltage regulated power supplies.

Fraction Collectors: Two ISCO fraction collectors with UV monitors.

Beckman Kintrac single beam spectrophotometer.

Radiometer pH Stat and Beckman pH meter

(list continues on page 3a)

11. Additional facilities required:

No additional space required.

(see Equipment in budget request)

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12. Biographical sketches of investigator(s) and other professional personnel (append):

See appended curriculum vitae for A. Cohen and T. Lo.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached reprints.

Source: https://www.industrydocuments.ucsf.edu/docs/ymll0000

Lyophilizer

Assorted refrigeration equipment and incubators

Tissue culture hood

Distillation and rotary evaporation equipment

Zeiss phase fluorescent photomicroscope

Isoelectric focusing apparatus

Hewlett-Packard model 9100 calculator

Photovolt densitometer

Mettler HT-20 micro balance and Mettler P1200 macro balance

Water baths

Other available facilities:

Packard gamma and beta isotope spectrometer (jointly owned with Dr. John Murray)

Gas-Liquid chromatograph (available from Dr. John Clements)

Cold Room Facilities (shared facilities with other University researchers located at S.F.G.H.)

Beckman HP119 amino acie analyzer

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14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s) even if no salary requested)

| | % time | Amount |
|---|---|---|
| Allen B. Cohen, M.D., Ph.D. | 30% | -0- |
| Theresa Lo, Ph.D. | 100% | -0- |

Technical

| | | |
|---|---|---|
| "to be recruited" Staff Research Associate II, Step 2 | 100% | $13,317 |

Sub-Total for A $13,317

B. Consumable supplies (by major categories)

| | | | |
|---|---|---|---|
| Enzymes | $2000 | Ampholytes (25 runs) | $ 500 |
| Substrates | $1500 | General Chemicals | $1500 |
| Column resins | $ 800 | Pipettes (vol. & micro) | $ 500 |
| Glassware | $1000 | Isotopes | $ 700 |
| Miscellaneous laboratory and office supplies | | | $1200 |

Sub-Total for B $ 9,700

C. Other expenses (itemize)

| | |
|---|---|
| Animals for immunization | $2000 |
| Blood for alpha-1-antitrypsin preps (200 units) | $5000 |
| Publication costs (reprints & page charges) | $1500 |
| Service contracts on equipment | $2000 |
| Travel ($500 for each professional) | $1000 |

Sub-Total for C $11,500

Running Total of A + B + C $34,517

D. Permanent equipment (itemize)

| | |
|---|---|
| ISCO Fraction collector and monitoring system | $3300 |
| Fluid pumps (2) | $ 800 |
| Haake Model FK water bath with heating, cooling, and external circulation | $ 750 |
| American Instruments Co. fluorocolorimeter | $1500 |

Sub-Total for D $6,350

E. Indirect costs (15% of A+B+C) E $5,178

Total request $46,045

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|---|---|---|---|---|---|---|
| Year 2 | 27,707 | 10,475 | 11,950 | 1500 (misc.) | 7520 | $59,152 |
| Year 3 | 29,862 | 11,313 | 12,425 | 1575 | 8040 | $63,215 |

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Yr. | Inclusive Dates |
|---|---|---|---|---|
| The role of the human alveolar macrophage in the production of emphysema | Council for Tobacco Research-USA, Inc. | 16,848<br>16,407<br>12,487<br>45,742 | 1<br>2<br>3 | April, 1971<br>to<br>March, 1974 |
| Alpha-1-antitrypsin: Does it inhibit an enzyme which causes emphysema? | USPHS, NHLI HL 14201 | 32,635<br>34,265 | 74<br>75 | June, 1971<br>to<br>June, 1976 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | | Inclusive Dates |
|---|---|---|---|---|
| The genetically determined biochemical defect in patients with emphysema and alpha-1-antitrypsin | USPHS, NHLI HL 06285 | 80,000 | 75 | July, 1975<br>to<br>July, 1982 |
| (See Budget Commentary, item #1 regarding this grant.) | | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Allen B. Cohen, M.D., Ph.D.

Signature Allen B Cohen Date 1-10-74

Telephone 415 (Area Code) 648-8200 (Number) ext.273 or 527 (Extension)

Checks payable to

Regents of the University of Calif.

Mailing address for checks

1487 Fourth Avenue

San Francisco, Calif. 94143

Responsible officer of institution

Typed Name Ms. Sue Clark

Title Program Coordinator
Gifts & Endowments Office

Signature Sue Clark Date 1/22/74

Telephone 415 (Area Code) 666-2047 (Number) (Extension)

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# BUDGET COMMENTARY

1. As shown on page 5 of the application, a grant submitted to the National Institutes of Health is pending. The grant is similar to this application but would start one year later if it is awarded. If both grants are funded to the extent requested, the renewal of the Tobacco Research Council grant will not be requested for the second and third years.

2. On his site visit last Fall, 1973, Dr. Gardner said that those investigators working on an April 1st grant year could be awarded grants beginning April 1st. If this is possible, I would appreciate having the April 1st starting date, with appropriate adjustment to the 12 month budget submitted.

3. Dr. Theresa Lo's salary will be paid by a Cardiovascular Research Institute Postdoctoral Traineeship until July, 1975. After this date Dr. Lo will continue to work as part of our permanent staff. During the entire time the experiments described in this protocol will occupy her full time. However, it will be necessary to pay her salary from this grant subsequent to July, 1975, and this is included in the Year 2 salary budget (salary, $11,325 plus staff benefits, $1,699 = total $13,024).

4. Fraction Collector and Monitoring System: This laboratory utilizes two fraction collectors nearly full time. In order to perform the numerous peptide and heterosaccharide separations required by the protocol, another fraction collector and monitor will be necessary. The equipment requested in this application interfaces with our existing fraction collectors and monitors from ISCO. They were selected because the dual beam optical unit has the sensitivity which will be required for the small amounts of ZZ protein which will be available and because the optical unit also reads absorbance at wave lengths necessary for monitoring ninhydrin reactions.

5. Fluoro-colorimeter: This instrument is necessary for measuring the extremely small amounts of protein from thin layer chromatograms, polyacrylamide gels, etc. With this instrument, using the method of Bohlen and his colleagues (Arch. of Biochem. 155:213, 1973), proteins, polypeptides, or amino acids can be measured in the nanogram range. In addition, quantitative analysis of dansylated amino acids is possible.

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9. Details of experimental design and procedures.

# THE GENETIC DEFECT IN ALPHA-1-ANTITRYPSIN DEFICIENT PATIENTS

Allen B. Cohen, M.D., Ph.D.

A). Background and rational: AAT deficiency is a genetically controlled disease characterized by a predisposition to pulmonary emphysema and low levels of AAT in the blood. AAT is an alpha-1-globulin in serum which inhibits trypsin, chymotrypsin, porcine pancreatic elastase, plasmin (1), thrombin (2), proteases from human neutrophils (3) and human alveolar macrophages (4). The protein has a molecular weight of 52000 (1) contains 2, half-cystines per mole., and probably has one polypeptide chain (5). In 1965 Gross and his colleagues demonstrated that an emphysema-like pathologic appearance developed a short time after the injection of papain into the tracheas of guinea pigs (6). Erikson (7) suggested the hypothesis that proteolytic enzymes from granulocytes and macrophages may destroy the parenchyma of the lung in AAT deficient patients because these enzymes are not adequately inactivated. Most research on the etiology of emphysema in AAT deficient patients has focused on this hypothesis. However, many important alternative possibilities have been bypassed even though there was no scientific reason to exclude them. The purpose of this protocol is to explore some of these possibilities by attempting to determine which protein is coded for by the abnormal gene in patients with this disease.

Studies of the genetics of AAT deficiency were made possible by the experiments of Fagerhol who developed a method of acid starch gel-immunocrossed gel electrophoresis for demonstrating that AAT differed qualitatively as well as quantitatively in patients with this disease (8). This qualitative difference is demonstrated by electrophoresing serum in low pH buffers in hydrolyzed starch blocks. The strip of starch containing the alpha-1-globulins is removed and placed on a thin slab of agar on a glass plate. The agar is made up with alkaline buffers and contains antibody against AAT. The alpha-1-globulins are electrophoresed across the agar gel and the alpha-1-antitrypsin precipitated in a pattern of peaks which characterizes the phenotype of the individual. The results of these studies were compatible with the hypothesis that the phenotype of AAT is controlled by a series of codominant alleles and the genes from both parents are expressed in the phenotypic pattern (8). The phenotypic pattern designated Pi type (proteinase inhibitor type) MM, characterized by 6 peaks, is

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manifested by the alpha-1-antitrypsin from most people and the phenotype pi AA, characterized by 4 peaks, is manifested by AAT from individuals who developed emphysema at an early age (6,9,10). The relationship of other phenotypes to lung disease is not proven, and therefore, they will not be studied in these experiments.

While the differences between the electrophoretic patterns of AAT from individuals with Pi types MM and ZZ have been clearly shown, the functions of the gene which causes the differences between these two electrophoretic patterns have not yet been determined. (In the ensuing discussion two types of heterogeniety of AAT will be examined: heterogeneity between individuals of different phenotypes, and heterogeneity within a single person. The first type of heterogeneity is the subject of this protocol, and the second type of heterogeneity will be examined only to the extent necessary to determine the structural basis of the differences between phenotypes.) An understanding of the structural differences between AAT of different phenotypes may suggest the function of the abnormal protein. For example, if MM and ZZ protein differed only by a specific carbohydrate moiety, then the defective gene may code for a sugar transferase which adds sugar moieties to the completed polypeptide. This possibility was suggested by the experiments of Bell and Carrell (11). These investigators showed that during digestion of MM protein with neuriminidase, the phenotype of MM protein passes through a phase in which it resembles the ZZ protein on acid starch gel-immuno-crossed gel electrophoresis. They suggest that a sialic acid transferase deficiency in the cells which produce AAT may cause the difference in phenotypic pattern between the two proteins. Such a change in the carbohydrate portion of the AAT molecule could explain other observations made in AAT deficient patients. An alteration in the carbohydrate could produce a protein with a genetically controlled change in electrophoretic microheterogeneity similar to the heterogeneity seen in AAT. Schmid et. al. (12) showed that similar electrophoretic heterogeneity was caused by sialic acid residue in alpha-1-acid glycoprotein. Since some glycoprotein chemists have suggested that the carbohydrate moiety is critical to the transport of proteins from the cells in which they are synthesized (13), and since AAT is synthesized in the liver, a defect in the carbohydrate moiety of AAT might also explain why patients whose AAT has the ZZ phenotype have large quantities of AAT in their livers as determined by fluorescent antibody techniques (14,15).

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In addition to the possibility of increasing the understanding of the biochemical defect in AAT deficient patients, these experiments may suggest that AAT deficiency need not be the cause of emphysema in AAT deficient patients. If the genetic defect in these patients is an abnormal glycotransferase, or glycosidase, then other proteins may also have abnormalities in their carbohydrate moieties. The possibility of other defects in addition to AAT deficiency is suggested in a recent publication by Ward and Talamo (16). They demonstrated that patients who have AAT of Pi type ZZ also have a deficiency of an inactivator of chemotactic factor in their serum. At least three interpretations of these experiments are possible: 1) there is an additional genetically controlled protein deficiency in AAT deficiency in AAT deficient patients, 2)

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the inactivator of chemotactic factor is AAT (preliminary studies by Ward and Talamo suggest that the factor is not AAT), 3) AAT may normally inactivate an enzyme which destroys the chemotactic factor inhibitor. If the first of these possibilities is correct, there may be a family of proteins which are abnormal in AAT deficient patients, and the abnormalities may all be caused by the protein which is coded for by the gene which controls the AAT phenotypic expression. For example, if a sugar transferase which was coded for by the defective gene were not specific for AAT a family of proteins with defective carbohydrate chains may exist in these patients. Such a gene could even affect structural glycoproteins such as lung collagen or elastin directly and result in emphysema by this mechanism.

If, however, it can be shown that MM protein differs from ZZ protein because of a point mutation in the structural gene which codes for the primary sequence of AAT, then it would be likely that any defects in AAT deficient patients would be secondary to the deficiency in AAT since with the exception of minor ambiguities in the genetic code (17) one structural gene codes for only one polypeptide.

In summary, AAT in patients with Pi types MM and ZZ differs in two ways, first those with Pi type ZZ have much lower levels of AAT in their blood, and second, AAT manifests complex patterns of electrophoretic microheterogeneity which are characteristic of the genotype. Any postulated mechanism for production of these differences must account for both phenomena. The presence of low levels of antigenically cross-reactive material (CRM) in patients with Pi type ZZ suggests that their genetic defects by the classification of Boyer and his colleagues (18). These investigators infer from structural analyses of human hemoglobin variants and from enzyme variants in microorganisms that most CRM+ mutations involve only one amino acid residue out of hundreds in a typical protein sequence. The difficulty in classifying AAT deficiency in this group of CRM+ defects arises when one tries to define a mechanism for the production of a genetically controlled complex pattern of electrophoretic microheterogeneity as the result of a substitution of a single amino acid.

The success of these experiments is predicated on the knowledge that the gene which causes AAT deficiency also causes, either directly or indirectly, a structural change in the AAT molecule, and the hope that the elucidation of the basis of the structural differences between AAT from individuals of different genotype will suggest the function of the protein coded for by the abnormal gene. If the gene codes for the AAT polypeptide, then standard methods of protein biochemistry may lead to the detection of this abnormality, and may permit interpretation of the nature of the defect in the AAT structural gene. If, however, the gene controls the carbohydrate moiety as in blood group specific glycoproteins, then study of the structure of the carbohydrate moiety may permit insight into the protein which controls the abnormality. These studies will be similar in many ways to studies which elucidated the genetically controlled differences between blood group glycoproteins.

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B). Experimental Design and Procedures: The purpose of this section is to describe the experiments which will provide the data necessary to reject either hypothesis 1 or hypothesis 2 stated earlier. Since all of the patients with Pi ZZ have very low levels of AAT in their blood and almost all of them develop emphysema at an early age, it is likely that some genetically controlled structural characteristic of ZZ protein causes the ATT to occur in the serum in low concentration. The purpose of these experiments is to determine the basis of the structural differences between MM and ZZ glycoproteins.

The structures of glycoproteins are probably determined by at least two genes (19). One of the genes determines the primary sequence of the amino acids in the polypeptide chain whereas the others are thought to control the heterosaccharide structure through enzymes which function as sugar transferases of variable specificity. While the microheterogeneity of some proteins and glycoproteins has been related to variation in the number of charged inorganic groups, desamido forms, or acetylated residues (20), addition of these groups has not yet been shown to be under genetic control. Therefore, there are three likely possibilities to be investigated: 1) the differences in electrophoretic microheterogeneity between MM and ZZ protein are related only to differences in the protein moiety, 2) they are related only to differences in the carbohydrate moiety, or 3) they are related to difference in both protein and carbohydrate moieties.

Possibility 1: If MM protein can be shown to differ from ZZ protein only by one amino acid and if the amino acid substitution can be related to a substitution of a single base in the genetic code (a point mutation), then Hypothesis 1 would be proven, unless ambiguity in the mammalian genetic code can be shown (21, 22). In addition, if MM and ZZ proteins differ in their polypeptide structure, other genetic mechanisms such as gene duplications, gene deletions, etc. or the possibility that heterogeneity is caused by the action of proteolytic enzymes on precursors forms will be considered.

Possibility 2: If, alternatively, MM protein can be shown to differ from ZZ protein by specific types or amounts of carbohydrate moieties only, then it can be suggested that a specific glycotransferase or glycosidase differs in the cells which synthesize different phenotypes. To prove Hypothesis 2 it would be necessary to demonstrate the alteration in the glycotransferase or glycosidase activity in tissues which synthesize AAT.

Possibility 3: If both carbohydrate and protein moieties were abnormal, the nature of the structural differences might suggest a mechanism for production of the differences. For example, if the primary sequence of amino acids in AAT showed an alteration in an amino acid residue which was the site of attachment of a carbohydrate chain, then the differences between phenotypes might still be related to a point mutation in the gene coding for the primary sequence of amino acids of AAT.

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The two hypotheses cannot be resolved by simply removing the carbohydrate from AAT and analyzing the polypeptide and carbohydrate moieties to determine the structural basis for the differences between MM and ZZ proteins. This direct approach to differentiating the two alternative hypotheses is not a practical approach because the methods for removing carbohydrates from proteins are poor and suffer from major inadequacies and complicating side reactions, and because there are no simple and well defined methods for sequencing heterosaccharides. Finally, the structural basis for the microheterogeneity within a single phenotype may have to be resolved before the structural basis of the genetically determined differences between differenct phenotypes can be determined. The approaches necessary to circumvent these problems and some of the factors which are expected to complicate the experiments are outlined in the Methods section.

General Methods: Enzyme assays: enzyme reaction rates will be measured with a Radiometer pH stat by a potentiometric method when nonchromogenic substrates are employed or with a kinetic recording spectrophotometer when chromogenic substrates are employed. Active sites of typsin and chymotrypsin will be titrated with p-nitrophenyl-p-quanido benzoate HCl by the method of Chase and Shaw (23). Other enzymes used in analysis will be titrated for active sites when reliable active site titrants are available. Concentrations of enzymes for which no active site titrants are available will be determined by their absorbance at 280 nm.

Since trypsin and chymotrypsin are stoichiometrically inhibited by equimolar concentration of AAT (1) concentration of active sites will be determined by titration with these two enzymes.

Purification of AAT: AAT of MM phenotype will be purified by the method of Cohen and Fallat (24). THis method yields sufficient AAT to perform functional studies (25,26). In addition, this is the only published method which yields AAT which is phenotypically unchanged from AAT in whole serum (21). This characteristic is necessary to the performance of these studies.

Schema for analyzing the genetically controlled structural differences between MM protein and ZZ protein:

Problem I: Purification of phenotypically unaltered of ZZ phenotypes.

Methods will be developed to prepare phenotypically unchanged AAT of ZZ phenotype. Several methods will be tried: 1) Method of Cohen and Fallat (24) for preparing AAT of MM phenotype, 2) Method of Crawford for preparing AAT from normal serum, (the integrity of the phenotype has been suggested but not documented), 3) antibody affinity columns eluted with thyiocyante (27) (Hydrogen ion elution will not be used because of the pH liability of AAT), 4) Enzyme affinity columns. (While such columns have been unsuccessful when trypsin and chymotrypsin were used, our recent investigations of the interaction of

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AAT with elastase suggests that the dissociation constant of AAT and elastase is higher than the dissociation constant of AAT with trypsin and chymotrypsin (26), therefore elution of AAT with a strong inhibitor of elastase such as paraoxon will be tried).

Problem II: Determining the number of different polypeptide chains among the components of a single phenotype.

If each of the component peaks of AAT in a single phenotype from one individual represents a different polypeptide chain, then the biochemical differences between MM and ZZ proteins must be analyzed on components from each phenotype which have the same polypeptide structure. Therefore, Problem II is to determine how many different polypeptide chains there are among the components of a single phenotype.

A partial resolution to this problem may be accomplished by end group analysis of phenotypically unchanged, purified MM and ZZ proteins by the dansylation method (28). Crawford (5) determined that the only end group in his preparation of MM protein was a glutamic residue. Isoelectric focusing of the purified protein resulted in a pattern which resembled the MM phenotype but the observation was not confirmed by the standard method of Fagerhol (18). Two other types of data suggest that the components of a single phenotype share the same polypeptide chain. All of the protein in purified AAT is active in the inhibition of trypsin and chymotrypsin (24) and each of the components share similar antigenic determinents with each other and with components of other phenotypes (8).

Further resolution of Problem II can be achieved by purifying the components of a single phenotype and demonstrating that the amino acid analysis of each component is idnetical to that of the other components within the error of the method which is about 3%. Since Crawford has shown that a pattern similar to the phenotype heterogeneity can be developed with analytic isoelectric focusing of purified MM protein it is possible that the components can be purified by preparative isoelectric focusing.

Problem III: Removing carbohydrate side chains.

Attempts will be made to eliminate the differences between MM and ZZ proteins by digesting the purified AAT of each phenotype with enzymes which only cleave carbohydrates or amino acid-carbohydrate bonds. If differences between MM and ZZ proteins can be eliminated by attacking the carbohydrate only, then two further possibilities must be differentiated: 1) there is an abnormality in heterosaccharide structure only, or 2) there is an abnormality in a serine, threonine, or asparagine residue at a point of attachment of a carbohydrate chain in AAT. There are at least three advantages in eliminating the differences between MM and ZZ phenotypes with enzymes which act on the

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carbohydrate moiety only. First, an abnormality in only one of the three carbohydrate binding amino acids could account for the differences between MM and ZZ proteins. Second, if a defect in the primary sequence of AAT can be ruled out, then the type of enzyme used to eliminate the differences between MM and ZZ protein may suggest the function of the abnormal protein which is coded for by the gene which controls the AAT phenotypic expression. For example, if the differences are eliminated by neuraminidase, then the abnormal protein in AAT deficient patients is likely to be a sialic acid tranferase. Finally, if the differences between MM and ZZ proteins are eliminated by an enzyme which cleaves carbohydrate chains, then an analysis of the differences between the chains cleaved from MM and ZZ proteins may suggest the function of the abnormal protein coded for by the AAT gene.

Two types of sugar cleaving enzymes are of particular interest, neuraminidase and N-acetyl-alpha-D-Galactosaminidase. Neuraminidase, an enzyme which cleaves terminal sialic acid residues from the carbohydrate chains, is important because Bell and Carrell (11) have claimed to change an AAT protein with MM phenotype as determined by Fagerhol's method (8) into a protein with ZZ phenotype by partial digestion with neuraminidase. These investigators have not ruled out the possibility that the modified MM may resemble ZZ only superficially. Further proof would consist of showing that complete digestion of MM and ZZ proteins with neuraminidase would yeild AAT which still indentical in acid starch gel.

Since there is some evidence to suggest that an attack on sialic acid residues will obliterate phenotypic differences between MM and ZZ proteins, this experiment will be performed first. Ideally, in order to demonstrate that phenotypic differences between MM and ZZ protein are due to the carbohydrate moiety of AAT, the carbohydrate moiety should be totally removed and the phenotypes should then be re-examined. However, cleavages of the carbohydrate from proteins is a complicated task. Carbohydrates are attached to mammalian glycoprotein by three main types of bonds. In the glycosylamine linkage, involving the amide group of asparagine so far only N-acetyl-glucosamine has been found to be the glycosyl connecting unit (Gly-Nac-Asn). The amino sugar linked O-glycosidically to peptide-bonded serine or threonine residues in glycoproteins seems to be invariably N-acetyl-galactosamine (29). The Gly-Nac-As bond is resistant to enzymes in whole glycoproteins and to mild acid and alkaline hydrolysis procedures but the O-glycosidic linkage involving the hydroxyl groups of serine and threonine can be cleaved in most glycoproteins and to mild acid and alkaline hydrolysis procedures but the O-glycosidic linkage involving the hydroxyl groups of serine and threonine can be cleaved in most glycoproteins with the enzyme N-acetyl-alpha-D-Galactosaminidase, after the glycoprotein is predigested with neuraminidase (30). Therefore, if the conclusions of Bell and Carrell (11) cannot be verified as outlined above, digestion serially with neuraminidase and N-acetyl-alpha-D-Galactosamine will be carried out on MM and ZZ proteins. The phenotypes

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will then be re-examined to determine if the differences have been eliminated. If the single or the dual enzyme digestion of MM and ZZ protein can abolish their differences, then experiments will continue with Problem IV.

Problem IV: Differentiation of a primary defect in carbohydrate structure from a defect in carbohydrate structure caused by a defection in amino acid sequence in AAT.

To determine if an amino acid defect exists in MM and ZZ proteins, the respective proteins will be cleaved into peptides. The peptides will be separated by column chromatographic methods, and amino acid analysis will be carried out on the small peptides (see appendix). Special attention will be given to carbohydrate containing peptides and the amino acid-carbohydrate linkages will be determined. If MM and ZZ differ by a single small peptide, isolation and sequencing of the peptide will be attempted by the Edman degradation method (31).

If only the carbohydrate moieties are abnormal and if the abnormality suggests a defect in a specific sugar transferase in the cells which synthesize AAT, then the absence of such a sugar transferase will be proven by examination of tissues from subjects with AAT phenotypes MM and ZZ. For example, if the difference between MM and ZZ proteins is determined only by terminal sialic acid residues as suggested by Bell and Carrell, then a sialic transferase will be sought in tissues from subjects with AAT phenotypes MM and ZZ. The experiments will be carried out using experimental designs which are similar to those of Ziderman. They showed that the genetically controlled differences between types A and B blood group substances were determined by difference in sugar transferases in certain tissues. Tissues were tested for glycosyl transferases of approporaite specificity. Both low molecular weight oligosaccarides of known structure and macromolecular glyco-proteins were used as sugar acceptors; nucleotide diphosphate sugars used as sugar donors and the monosaccharide component carried the radioactive marked. Finally, it may be possible to convert one phenotype to another with the appropriate sugar transferase, sugar moiety, and sugar donor.

Problem V: Analysis of the biochemical basis for phenotypic differences between MM and ZZ proteins if the differences cannot be eliminated by removing sugar residues.

If the differences between MM and ZZ proteins cannot be eliminated by glycosidases or glycosaminidases then the problem of defining the differences between these proteins is more complicated. The carbohydrate chains which are different will likely be difficult to separate in tact from the protein moieties, and the possible amino acid defects in the primary sequence of AAT are not limited to those amino acids which serve as attachment points for the carbohydrate chains.

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AAT will be cleaved at methionyl residues with CNBr (35). Then in order to facilitate further digestion of AAT into peptides, disulfide bonds will be reduced with dithiothreitol by the method of Cleland (36) and S-carboxy methylated (37). At each step peptide fragments will be separated from each other and the carbohydrate containing peptides will be separated from the non-carbohydrate containing peptides. Enzymatic digestion will be carried out on neuraminidase treated peptides to facilitate complete digestion (38). Enzymes which may be used include trypsin, chymotrypsin, papain, subtilisin, etc. Those peptides of both classes which are identical between MM and ZZ proteins will not be further analyzed. Carbohydrate free peptides from MM protein will be separated by ion exchange chromatography (39), analyzed for AA content, and matched with peptides in ZZ proteins for differences (see appendix). Particular attention will be paid to single amino acid substitutions which could be caused by the AAT controlling gene.

In order to analyze the carbohydrate containing peptides first the peptide moiety will be separated from the carbohydrate moiety so that the polypeptide can be analyzed by the methods described in the preceding paragraph. Removal of the carbohydrate chains from seryl or threonyl bonds will probably be achievable with N-acetyl-alpha-D-Galactosaminidase (40) or by alkaline hydrolysis (41). However, removing in-tact heterosaccharide chains from the aspartyl bond is more troublesome. Partial acid hydrolysis may have some success (42), however, this treatment may also cleave some peptide bonds.

For isolation of the carbohydrate chains, the peptide will be digested away as completely as possible with proteolytic enzymes. Carbohydrates will be separated from peptides and free amino acids on ion exchange columns (43) or on conconavalin columns (44). The connecting amino acids will be cleaved with the enzymes, 4'-L-aspartyl glycosylamine amido hydrolase or acetyl-alpha-D-Galactosaminidase (see appendix) and the connecting amino acid identified. Further clarification of differences in carbohydrate chains between MM and ZZ proteins poses a problem which cannot be solved in advance since there are no reliable methods for sequence determination of these chains. Some success has been achieved with a combination of gentle acid hydrolysis and specific glycosidases, but the methods are not universally applicable (45). If the differences between MM and ZZ proteins can be localized to the carbohydrate moieties only then another possible approach would be to examine tissue from ZZ patients for absences of all the specific sugar transferases which are involved with synthesis of AAT. Since AAT contains only hexoses, hexosamines, acetylneuraminic acid, and fucose (46), seeking transferases for each of these sugars may be a managable experiment.

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## Appendix 1

Hydrolysis of glycoproteins and amino acid analysis: In order to reduce the loss of cystine, methionine and tyrosine and artifact information due to oxidation of amino acids, amino acid hydrolysis will be carried out by the method of Blackburn (47). The protein will be dissolved in 6NHCl in a pyrex container, frozen, then the glass container will be evacuated to below 0.05 mm of Hg. The protein solution will then be thawed for degassing, re-frozen, and sealed. To reduce the interaction of carbohydrate degradation byproducts solutions of glycopeptide will be kept below 0.1%. Samples will be heated in an oven at 110°C for 24, 48, and 72 hour periods, the top of the tube removed and the tube will be evaporated to dryness. Before analysis the residue will be dissolved in water and brought to pH 6.5 with phosphate buffer and allowed to stand for 4 hours to allow oxidation of cysteine to cystine. It will then be prepared for application to the amino acid analyzer.

Analysis of amino acids will be performed on a Beckman HP 119 amino acid analyzer equipped with an Autolab integrator using Beckman Spinco AA-15 resin at a constant temperature of 52°C. Tryptopan will be determined by hydrolysis in p-Tolulenesulfonic acid by the method of Liu (49). Methionine will be determined by oxidizing the peptide to methionine sulphoxide by the method of Stegeman (50) before analysis. Values for serine and threonine will be extrapolated to zero time from the hydrolyzated heated for 24, 48, 72 hours. Values for isoleucine and valine will be determined by hydrolysis for 72 hours. Cystine will be determined on separate paptide samples as cysteic acid after performic acid oxidation (51).

## Appendix 2

Preparation of peptides for mapping: Denatured, reduced and carboxymethylated MM or ZZ proteins will be cleaved first by cyanogen bromide (35), and the purified fragments will be digested with trypsin (52). The peptides obtained at each step will be isolated by gel filtration or ion exchange chromatography. The theoretical yield of peptides from the procedures would be 46. At both steps, attempts will be made to isolate TCA insoluble peptides by precipitation with 5% TCA. If TCA insoluble peptides are found, further cleavage of peptides may be required by other proteolytic enzymes before they are further analyzed.

Initial separation of peptides will be carried out with anion exchange chromatography with Dowex 1-x2, using the method of Fantasu (53). The peptides will be eluted with a gradient starting with a buffer which is 1% collidine, 1% pyridine, and 6.25 mM in acetic acid (pH=8.2) and progressive drops in Ph until that of 5N acetic acid. Peptides will be further separated on a cation exchange column mounted on the amino acid analyzer using Beckman-Spinco resin PA-35. The chromatogram will be developed with a Buchler varigrad

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gradient maker which will employ a starting buffer of 0.2M pyridine acetate buffer at pH=3.1, and a second buffer of 2.0M pyridine acetate buffer at pH=5.0 as described by Jones (39). These methods are first approximation and final methods are first approximation and final methods will be determined by experimentation.

Appendix 3

Purification of alpha-D-galactosaminidase: Alpha-D-galactosaminidase will be purified from beef liver by the method of Weissman and Hinrichsen (40). These investigations achieved a 700 fold purification of the enzyme using ammonium sulfate fractionation, column chromatography on DEAE-cellulose, and Sephadex G-150 sequentially.

Appendix 4

Purification of 4'-L-Aspartylglycoslyamine amido hydrolase (AAH): Purification of this enzyme will be performed by the method of Makino (54), from hog serum. The methods used sequentially, ammonium sulphate precipitation, CM-cellulose chromatography, DEAE-cellulose chromatography, and calcium phosphate chromatography. The material recovered is still heterogeneous, but it contains no proteolytic or glycosidic activities toward glycoproteins.

Appendix 5

Carbohydrate analysis: The procedure to remove carbohydrate from seryl and threonyl residues by alkaline hydrolysis or enzymes and from aspartyl residues by alkaline hydrolysis or enzymes and from aspartyl residues by acid hydrolysis and the procedure to separate the free carbohydrate chains have been referenced in the text (40,41,42,43). Analysis of the carbohydrate chains will be performed by gas-liquid chromatography after methanolysis by the methods of Clamp, Bhatti and Chambers (55).

Preliminary experiments will include the preparation and analysis of carbohydrate chains from glycoproteins of known structure in order to verify the method. Samples of suitable weight will be combined with sufficient internal standard to give peaks of similar height. 0.5 ml of 1.5N methanolic HCl will be added to each dried ampoule. The ampoules will be sealed under nitrogen and heated at 90°C for 24 hours or varying times and temperatures when indicated. After cooling and neutralization re-N-acetylation will be carried out by addition of acetic anhydride. Supernatants will be pooled and acetic anhydride removed with rotary evaporation at 25°C. The trimethylsilyl derivatives will be prepared by adding 50 ul of TriSil-Z to the dried material in a screw capped vial. The vial will immediately be sealed and the reaction will be allowed to proceed for 30 minutes at 25°C. A 2 ul aliquot of the

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sample will be injected into the GLC. The conditions of chromatography are described by Clamp, Bhatti, and Chamber (55). Conditions of hydrolysis, re-N-acetylation, and trimethylsilylation will be varied to give maximal yields.

Appendix 6

Unpaid Consultant: Since the experience in this laboratory with carbohydrate analysis in glycoproteins is limited, consultative services have been arranged with Dr. Johnathon Doxon, Ph.D. who is a glycoprotein chemist in the Hormone Research Laboratory and who is actively performing carbohydrate analyses on glycoproteins with gas-liquid chromatography.

C). Significance of this work: Since the occurance of AAT deficiency in some patients with emphysema is one of the few clues available to the nature of the biochemical processes in human emphysema, an understanding of these processes may lead to modes of therapeutic intervention. This section will attempt to answer two questions: first, what is the importance of AAT deficiency to emphysema from all causes in this country, and second what contribution will the answers to the questions posed in this protocol make to the understanding, treatment and control of AAT deficiency.

Two questions must be answered in order to determine the importance of AAT deficiency to human emphysema. First, what proportion of the population with abnormal AAT genes get emphysema as a result of these genes, and second is there a link between the the AAT related emphysema and the more common form of emphysema seen in smokers? The answer to the first question is controversial. While all investigators agree that only a rare patient with pi type ZZ survives to the age of 50 without emphysema (7). Estimates of the incidence of lung disease in subjects with pi type MZ vary from nil in Eriksson's study to substantial in the work of Lieberman (56) and of Kueppers and Larson (57), who estimated that 26 and 29% respectively of the patients with chronic obstructive pulmonary disease had AAT with pi types ZZ or MZ. The incidence of the Z gene in Americans has been estimated from 0.01 to 0.016 (9, 57). In addition, if Eriksson's estimate of the prevalence of patients with pi type ZZ of 0.00057 (7) is also found in Americans, then more than 140,000 Americans are at high risk of developing emphysema related to AAT deficiency. In addition, the data of Black and his colleagues (58) suggests that nonsmoking homozygotes may get the emphysema about a decade later than smoking homozygotes. Therefore, smoking may exacerbate the propensity of subjects with pi type ZZ to get emphysema or perhaps the two forms of emphysema may be produced through a common biochemical mechanism. One possible common pathway was suggested by Cohen (4). If there is a common pathway, then drugs developed to prevent

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emphysema in AAT deficiency may also prevent emphysema in smokers who get emphysema.

Evidence was reviewed in the introduction to this protocol which shows that most investigaors who study AAT deficiency work on the premise that the low levels of AAT are the proximate cause of the emphysema in deficient patients, and that the emphysema is caused by the unrestrained action of one or more enzymes on pulmonary tissues. This protocol presents evidence which suggests that defects other than the AAT deficiency are present in these patients and suggests experiments which might either show that these other defects are secondary to the deficiency in AAT or show how to determine the biochemical nature of the other defects if the other defects are not secondary. The possibility that other defects occur in the same people and that one of the other defects may cause the emphysema which these people develop must be eliminated before investigations in this field become completely confined to AAT.

D). Probable Future Direction of this Work: If the weight of evidence suggests that alpha-1-antitrypsin is the protein coded for by the abnormal gene in deficient patients, then it would be likely that all of the other biochemical lesions would be secondary to this lesion. This information would suggest that addition of alpha-1-antitrypsin-like activity to the blood of deficient patients might prevent the emphysema which they develop. Therefore, methods for accomplishing this goal would be investigated. The goal might become possible through several possible routes. Our SCOR B-1 research on the biochemical mechanism of the active sites of alpha-1-antitrypsin may permit the design of drugs which would replace the function of the missing inhibitor. Other lines of investigation in the proposed research may suggest methods of releasing alpha=1-antitrypsin from livers of deficient patients. In addition, many other investigators are exploring other means for accomplishing these ends.

If, however, this research suggests that biochemical lesions other than the low levels of alpha-1-antitrypsin may exist in these patients then future research would explore these other lesions to determine if one of them might be the cause of emphysema in alpha-1-antitrypsin deficient patients.

E). Relationship to Dr. Cohen's SCOR Project: Dr. Cohen's SCOR project is related to the determination of the mechanism of action of the inhibitory sites on AAT and this protocol is related to the determination of the genetic differences between AAT of pi types MM and ZZ. Since patients with pi type ZZ have trypsin inhibitory capacities commensurate with their levels of AAT,

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the active site of AAT is probably functional in ZZ protein. Therefore, it is unlikely that experiments in one protocol will be useful in the goals of the other protocol. In fact, the procedures to be employed in this protocol will very likely destroy the inhibitory site on AAT. Therefore, there will be little if any overlap between the protocols.

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References


1. Heimberger N. and H. Haupt, Klin, Wschr., 44:1196, 1966
2. Rimon A., Y. Shamash,and B. Shapiro, J. Biol. Chem., 241:5102, 1966
3. Herman G., P. Miescher, Int. Arch. Allerg., 27:346, 1965
4. Cohen A.B., J. Clin. Invest. In Press.
5. Crawford I., Arch of Biochem. & Biophys., 156:215, 1973
6. Gross P., E. Pfitzer, E. Tolker, M. Babyak and M. Kaschak, Arch. Environ. Health, 11:50, 1961
7. Eriksson S., Acta. Med. Scand., 175:197, 1964
8. Fagerhol M.K. and T. Gedde-Dahl, Human Heredity, 19:354, 1969
9. Kueppers F., W. Briscoe, Bearn A., Sci., 146:1678, 1964
10. Falk G.A. and W. Briscoe, Ann. Intern. Med., 72:427, 1970
11. Bell O.F. and R. Carrell, Nature, 243:410, 1973
12. Schmid K., J. Binette, S. Kaiyama, V. Pfister, and S. Takashi, Biochem. 1:959, 1962
13. Melchers, F.and P. Knopf, Cold Spring Harbor Symp. Quant. Biol. 32:255, 1968
14. Sharp H.L., Hosp. Pract., 6:83-92, 1971
15. Sharp H.L., R. Bridges, W. Krivit, J. Lab. Clin. Med., 73:934, 1969
16. Ward P.A. and R. Talamo, J. Clin. Invest., 52:516, 1973
17. Lehmann H. and R. Carrell, Brit. Med. Bull., 25:14, 1969
18. Boyer S.J., D.C. Siggers, and L.J. Kruegers, Lancet 2:654, 1973
19. Gibbons R.A., Ch. 3, Sect. 1, Glycoproteins pp. 36, Ed. A. Gottschalk, Elseverier Publishing Co., N.Y., 1972
20. Harfenist E., J. Am. Chem. Soc., 75:5528, 1953
21. Lehmann H. and R.W. Carrell, Brit. Med. Bull., 25:14, 1969
22. Lehninger A., Biochemistry Worth Publishing, N.Y. 1970
23. Chase T. and E. Shaw, Biochem. Biophys. Res. Commun., 29:508, 1967
24. Cohen A.B. and R. Fallat, Biochem. Biophys. Acta. In Press.
25. Cohen A.B., J. Biol. Chem. In Press.
26. Cohen A.B., Submitted to J. Biol. Chem.
27. Dandliker W.B., and V. DeSaussure, Immunochem. 5:357, 1968
28. Gray W., Sect. 12 Methods in Enz., 11:139, 1967
29. Neuberger A., A. Gottschalk, R. Marshall and R. Spiro, Ch. 4, Sec. 2, Glycoproteins pp. 452. Ed. A. Gottschalk
30. Gottschalk A., H. Schauer,and G. Uhlenbruck, Physiol. Chem. 352:117, 1971
31. Edman P., Acta. Chem. Scand., 10:761, 1956
32. Ziderman D., S. Gompertz, Z. Smith, and W. Watkins, Biochem. Biophys. Res. Commun., 29:56, 1976
33. Kobata A., E. Grollman, and V. Ginsburg, Arch. Biochem. Biophys., 124:609, 1968
34. Race C., D. Ziderman, and W. Watkins, Biochem. J.,107:733, 1968
35. Gross E. and B. Witkop, J. Biol. Chem. 237:1859, 1962
36. Cleland W., Biochem., 4:480, 1964


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Source: https://www.industrydocuments.ucsf.edu/docs/ymll0000

References (continued)


37. Edelman G., W. Gall, M. Waxdal, and W. Konigsberg, Biochem. 7:1950, 1968
38. Gottschalk A., S. Fazekas, and de St. Groth, Biochem. Biophys. Acta., 43:513, 1960
39. Jones R.T., Methods of Biochemical Analysis, 18:205, 1970
40. Weissman B., and D. Hinrichsen, Biochem. 8:2034, 1969
41. Bhavanandan V., E. Buddecke, R. Carubelli and A. Gottschalk, Biochem. Biophsy. Res. Commun., 16:353, 1964
42. Neuberger A., A. Gottschalk, R. Marshall and R. Spiro, Chap. 4, Sec. 2 Glycoproteins pp. 451, Ed. A. Gottschalk, Elsevier Publishing co., N.Y. 1972
43. Spiro R.G., J. Biol. Chem., 240:1603, 1965
44. Goldstein I., L. So, L. Yang, and Q. Callies, J. of Immunol., 103:695, 1969
45. Clamp J.R. and I. Johnson, Chap. 6. Sec. 3, Glycoproteins pp. 638. Ed. A. Gottschalk, Elsevier Publishing Co., N.Y., 1972
46. Schultze H. and J. Heremans, Molecular Biology of Human Proteins, Vol. 1, 1966
47. Blackburn S., Amino Acid Determination: Methods and Techniques, Marcel Dekker Co., N.Y., 1968
48. Gottschalk A., Ann. N.Y. Acad. Sci., 106:168, 1963
49. Liu T., Methods in Enzymology, 25:44, Ed. C. Hirs and S. Timasheff, 1972
50. Stegeman H.,Physiol. Chem., 319:87, 1960
51. Hirs C., J. Biol. Chem. 219:611, 1956
52. Smyth D.G., Section V-(24) Methods in Enzymology, 11:214, 1967
53. Fantasu G., Biochem., 3:1351, 1964
54. Makino M., T. Kojima, T. Ohgushi, and I. Yamashina, J. Biochem., 63:186, 1968
55. Clamp J.R., T. Bhatti, and R. Chambers, Chapt. 3, Sec. 6, Glycoproteins Elsevier Publishing Co. N.Y. pp. 300, 1972
56. Lieberman J., N. Eng. J. Med., 281, 1969
57. Kueppers F. and R.K. Larson, Sci., 165:899, 1969
58. Black L.F., R.E. Hyatt, and S.E. Stubbs, Am. Rev. Resp. Dis., 105:891, 1972


1003540004

Source: https://www.industrydocuments.ucsf.edu/docs/ymll0000